EXHIBIT 4

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Size Distribution and General Structural Features of *N*-Linked Oligosaccharides from the Methylotrophic Yeast, *Pichia pastoris*

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The secreted glycoproteins of *Pichia pastoris* contain more than 35% of their *N*-linked oligosaccharides as structures smaller than $Man_{14}GlcNAc_2$ (Man = mannose; GlcNAc = *N*-acetylglucosamine). On heterologous invertase produced in *P. pastoris*, approximately 85% of the oligosaccharides are in the size range $Man_{8-14}GlcNAc_2$. The structures appear to contain α-linked mannose. In addition, one-third of the structures contain net negative charge and can be radio-labelled *in vivo* with ^{32}P. The largest oligosaccharides isolated from *P. pastoris* are significantly shorter than the hypermannosylated structures typical of *S. cerevisiae*, indicating that the factors which influence the processing of *N*-linked oligosaccharides in *P. pastoris* are different from those which influence processing in *S. cerevisiae*. The smaller *N*-linked oligosaccharides synthesized by *P. pastoris* resemble high-mannose oligosaccharides synthesized by animal cells, and this finding increases the utility of *P. pastoris* as a host for the production of heterologous glycoproteins.

KEY WORDS — *Pichia pastoris*; glycoproteins; invertase; oligosaccharides.

INTRODUCTION

Pichia pastoris, a methylotrophic yeast, has recently been developed as an efficient system for the production of recombinant proteins (Cregg *et al.*, 1987; Cregg and Madden, 1988; Tschopp *et al.*, 1987a,b). In this yeast, heterologous proteins have been expressed at high levels both cytoplasmically and as secreted proteins (Cregg *et al.*, 1987; Cregg and Madden, 1988; Digan *et al.*, 1988; Tschopp *et al.*, 1977a,b). The *SUC2* invertase gene of *S. cerevisiae* has been expressed as a heterologous protein in *P. pastoris* and the glycosylated, fully active protein is secreted into the periplasmic space and growth medium at levels of 2·5 g/l (Tschopp *et al.*, 1987b). The heterologous invertase is synthesized as a 58 000 molecular weight polypeptide which becomes glycosylated at an average of nine or ten of 14 available glycosylation sites and has a relatively homogeneous, glycosylated molecular weight of 85 000–90 000 (Tschopp *et al.*, 1987b). Indirect experimental evidence and calculation have indicated that the average size of the oligosaccharides is $Man_{10}GlcNAc_2$ (Tschopp *et al.*, 1987b). This contrasts sharply with results obtained for invertase synthesized in *S. cerevisiae*, where marked heterogeneity in the size of individual oligosaccharide chains (Reddy *et al.*, 1988) and great variability in the molecular weight of the secreted glycoprotein are reported (Ziegler *et al.*, 1988).

We were interested in determining, experimentally, whether the oligosaccharides of invertase synthesized in *P. pastoris* were as small as calculated, whether this calculation reflected a uniformly sized population, and whether the glycosylation of the heterologous invertase reflected the general pattern of glycosylation in *P. pastoris*.

MATERIALS AND METHODS

Growth of cells and radiolabelling with ^3H-mannose

Pichia pastoris cells GS115 (Cregg *et al.*, 1985) transformed with the invertase expression vector pGS102 (Tschopp *et al.*, 1987b) were grown at 30°C in a minimal salt medium (Wickerham, 1946) containing 0·2 mM-ammonium sulfate and 2% glycerol to mid-log phase. Cells (10 OD^{600} units) were washed and resuspended in 10 ml of minimal medium containing 0·2 mM-ammonium sulfate and 0·5% methanol. After 1 h of incubation in a 25 ml

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shake flask, cells were centrifuged and the pellet was resuspended in 1 ml of 2 × minimal medium containing 0·4 mM-ammonium sulfate and 1% methanol. One ml of 2-[^{3}H]-mannose in sterile aqueous solution (1 mCi/ml; New England Nuclear) was added to the cell suspension. After 2·5 h of shaking at 30°C, the culture was centrifuged and the cell pellet was resuspended in the original methanol-containing medium for a chase period of 2·5 h at 30°C. For the 24-h labelling, a 1% methanol-containing culture was incubated for 1 h prior to addition of 1 ml of 2-[^{3}H]-mannose (1 mCi/ml; New England Nuclear). During the 24-h labelling, the cells grew three to four generations.

For phosphate labelling, *P. pastoris* cells were first grown in a 2% glycerol culture as described above. Cells (10 OD^{600} units) were washed and resuspended in 10 ml of low-phosphate medium (Stevens *et al.*, 1982) containing 0·5% methanol. Incubation in a 50 ml shake flask was continued for 3 h at 30°C. Cells were centrifuged and the cell pellet was resuspended in 1 ml of 2 × concentrated low-phosphate medium (Stevens *et al.*, 1982) containing 1% methanol. Sterile water (0·7 ml) and 0·3 ml ^{32}P-phosphate (3 mCi; 10 mCi/ml; New England Nuclear) were added, and the culture was incubated in a 30°C air shaker for 3 h.

S. cerevisiae cells SEY2102 (Emr *et al.*, 1983) transformed with invertase expression vector pRB58 (Carlson and Botstein, 1982) were grown at 30°C in minimal medium (Wickerham, 1946) containing 0·2 mM-ammonium sulfate, 20 mg/l histidine and leucine, and 5% glucose to mid-log phase. Cells (10 OD^{600} units) were washed with water and resuspended in 10 ml of minimal medium containing 0·2 mM-ammonium sulfate, 20 mg/l histidine and leucine, and 0·05% glucose. Cells were incubated for 1 h at 30°C to derepress for invertase (Carlson and Botstein, 1982; Schekman and Novick, 1982). Labelling with 2-[^{3}H]-mannose was for 2·5 h.

Cell fractionation and immunoprecipitations

Following *in vivo* labelling with 2-[^{3}H]-mannose or ^{32}P-phosphate, the cells were pelleted using a table-top centrifuge. The cell pellets were suspended in 0·3 ml sorbitol buffer (Tschopp *et al.*, 1987b), and 50 µl of Zymolyase (4 mg/ml; Miles Laboratories) was added. The suspension was incubated at 37°C for 1 h, after which the spheroplasts were centrifuged for 2 min in an Eppendorf centrifuge. The supernatant of this centrifugation is referred to as the periplasmic fraction. Immunoprecipitations of invertase from the periplasmic fraction and from the media were performed as previously described (Tschopp *et al.*, 1987b). Unlabelled invertase was isolated from growth media as previously described (Tschopp *et al.*, 1987b).

Enzymes and chemical treatments

Glycoprotein samples of 0·5 ml were desalted by spin dialysis using Sephadex G-10 (Pharmacia). The samples were treated with 40 µl Pronase (20 mg/ml; Calbiochem) in 0·15 M-Tris–HCl, pH8·0, 10 mM-$CaCl_2$, and 0·02% NaN_3 for 36–48 h at 50°C, with additions of 25 µl Pronase at 12, 24 and 36 h. The Pronase was autodigested for 1 h at 37°C prior to addition to the sample. Following Pronase treatment, the samples were boiled for 10 min, desalted and lyophilized, and then treated with 0·3 µg/ml endo-β-*N*-acetylglucosaminidase H (endoH; New England Nuclear) in 50–200 µl, 0·1 M-sodium citrate, pH 5.5, 0·02% NaN_3 for 18–24 h at 37°C. Digestion with 5–10 µg α-mannosidase (Boehringer Mannheim) was performed in 0·05 M-sodium acetate, pH 5·0, and 0·1 mM-$ZnCl_2$. Enzyme activity was confirmed at the end of the digestion using *p*-nitrophenyl-α-D-mannoside (Sigma). *Escherichia coli* alkaline phosphatase (500 µg/ml; Calbiochem) treatment was performed on 0·1–0·5 ml samples for 3–18 h in 0·05 M-Tris, pH 8·0, containing 20 mM-$CaCl_2$. Activity was confirmed using *p*-nitrophenyl-phosphate (Sigma). All enzyme reactions were terminated by heating the samples to 100°C for 5 min. Mild alkaline hydrolysis was performed using 1 M-$NaBH_4$ and 0·1 M-NaOH for 18 h at room temperature and was stopped by the addition of acetic acid. *In vitro* labelling of oligosaccharides with $NaB[^3H]_4$ (1·4 Ci/mmol; ICN) was essentially as described elsewhere (Brands *et al.*, 1985). A sample not treated with endoH was used as control.

Chromatography

Oligosaccharide samples were applied to Bio-Gel P-4, P-6, and/or P-10 columns (1 × 40 cm, each <400 mesh, Bio-Rad). The columns were equilibrated with 0·1 M-pyridinium acetate, pH 6·0, at a flow rate of 6 ml/h. Fractions of 0·45 ml were collected and monitored for radioactivity in EcoLite (WestChem) using a Beckman LS 1801 scintillation counter. The Bio-Gel columns were calibrated with reference oligosaccharides prepared as previously

described (Grinna and Robbins, 1979). Bovine serum albumin (0·5 mg) and 2-[^{3}H]-mannose were added to the samples as void volume (V_0) and inclusion markers (V_m, V_i) markers. K_D values were calculated as previously described (Grinna and Robbins, 1979). QAE-Sephadex (Pharmacia) chromatography was performed on a 1 × 2 cm column equilibrated in 2 mM-Tris base, pH 7·5. After applying the sample in approximately 2–5 ml volume, the column was washed with 2 mM-Tris base and then eluted stepwise with 10, 50, 200 and 500 mM-NaCl. Aliquots of the eluted fractions were monitored for radioactivity, pooled, desalted and lyophilized prior to chromatography on Bio-Gel columns.

RESULTS

Size and distribution of N- and O-linked oligosaccharides

Oligosaccharides released by endoH treatment of the soluble periplasmic glycoprotein and periplasmic invertase were each fractionated on Bio-Gel P-6 and P-10 columns. As seen in Figure 1, similar patterns in the size distribution of the mannose-labelled oligosaccharides were obtained. The oligosaccharides fractionated into two major size groups, with only minor amounts of oligosaccharides between the groups (Figure 1A, B, D, E). The small oligosaccharides ranged in size from $Man_8GlcNAc$ to approximately $Man_{14}GlcNAc$ and constituted a significant portion of the total oligosaccharide synthesized by *P. pastoris* (Figure 1A, D). The group of small $Man_{8-14}GlcNAc$ oligosaccharides isolated from the periplasmic glycoprotein comprised about 35% of the mannose label incorporated during *in vivo* labelling of 2·5 or 24 h (Figure 1A, B). For invertase, the amount of the small oligosaccharide fraction was more variable but generally appeared to be somewhat larger, comprising from 40 to 70% of the incorporated mannose label (Figure 1D, E). In every case, oligosaccharides of size $Man_{10-14}GlcNAc$ accounted for more than 60% of the total small oligosaccharide fraction. In similar experiments using *S. cerevisiae*, only 5–10% of the incorporated mannose label was found in the small oligosaccharide fraction (Figure 1B, E).

The large oligosaccharide structures synthesized by *P. pastoris* are larger than $Man_{>30}GlcNAc$ and are eluted in the void volume of Bio-Gel P-6 columns (Figure 1A, D). These large structures are, however, included in the separating volume of P-10 columns (Figure 1B, E) and are smaller than the large oligosaccharides of *S. cerevisiae* which are eluted in the void volumes of both the Bio-Gel P-6 (not shown) and P-10 columns (Figure 1B, E). Although the maximum size of the oligosaccharides was not determined, it is clear that the largest oligosaccharides synthesized by *P. pastoris* are truncated relative to the largest oligosaccharides synthesized by *S. cerevisiae*.

To obtain a quantitative measure of the distribution of the large and small oligosaccharides, *in vitro* sodium borohydride reduction was used to label only the reducing end of endoH-treated oligosaccharides. Using invertase purified from the growth medium of *P. pastoris*, it was determined that less than 6% of the total incorporated [^{3}H] counts were present in the large oligosaccharides, while more than 85% of the incorporated [^{3}H] counts were in oligosaccharides of size $Man_{8-14}GlcNAc$ (Figure 1F). More than 60% of these oligosaccharides were of the size $Man_{10-14}GlcNAc$. From this distribution and the assumption that, on the average, there are nine oligosaccharide chains on each invertase, it can be inferred that two to four chains are $Man_{8,9}GlcNAc_2$, five or six chains are in the size range $Man_{10-14}GlcNAc_2$, and only one chain is $Man_{>30}GlcNAc_2$. This pattern clearly demonstrates the preponderance of short-chain oligosaccharides on invertase secreted by *P. pastoris* and also demonstrates that the population of oligosaccharides is relatively homogeneous. A median chain length of $Man_{10}GlcNAc$ was calculated from this profile.

To determine whether O-linked oligosaccharides are significant components of the total glycoprotein secreted by *P. pastoris*, Pronase-treated samples of the mannose-labelled periplasmic fraction were further treated by mild alkaline hydrolysis and analysed by gel filtration. The mild alkaline hydrolysis produced only slight changes in the pattern of Pronase-treated oligosaccharides, indicating that mannose-containing, O-linked oligosaccharides, although present, are not major components of the total soluble glycoprotein of *P. pastoris* (Figure 1C). Invertase secreted by *P. pastoris* was also treated with Pronase and by mild alkaline hydrolysis. The gel filtration pattern of the Pronase-treated sample did not change following the mild alkaline treatment, indicating that O-linked oligosaccharides are not major components of this protein when synthesized by *P. pastoris* (data not shown).

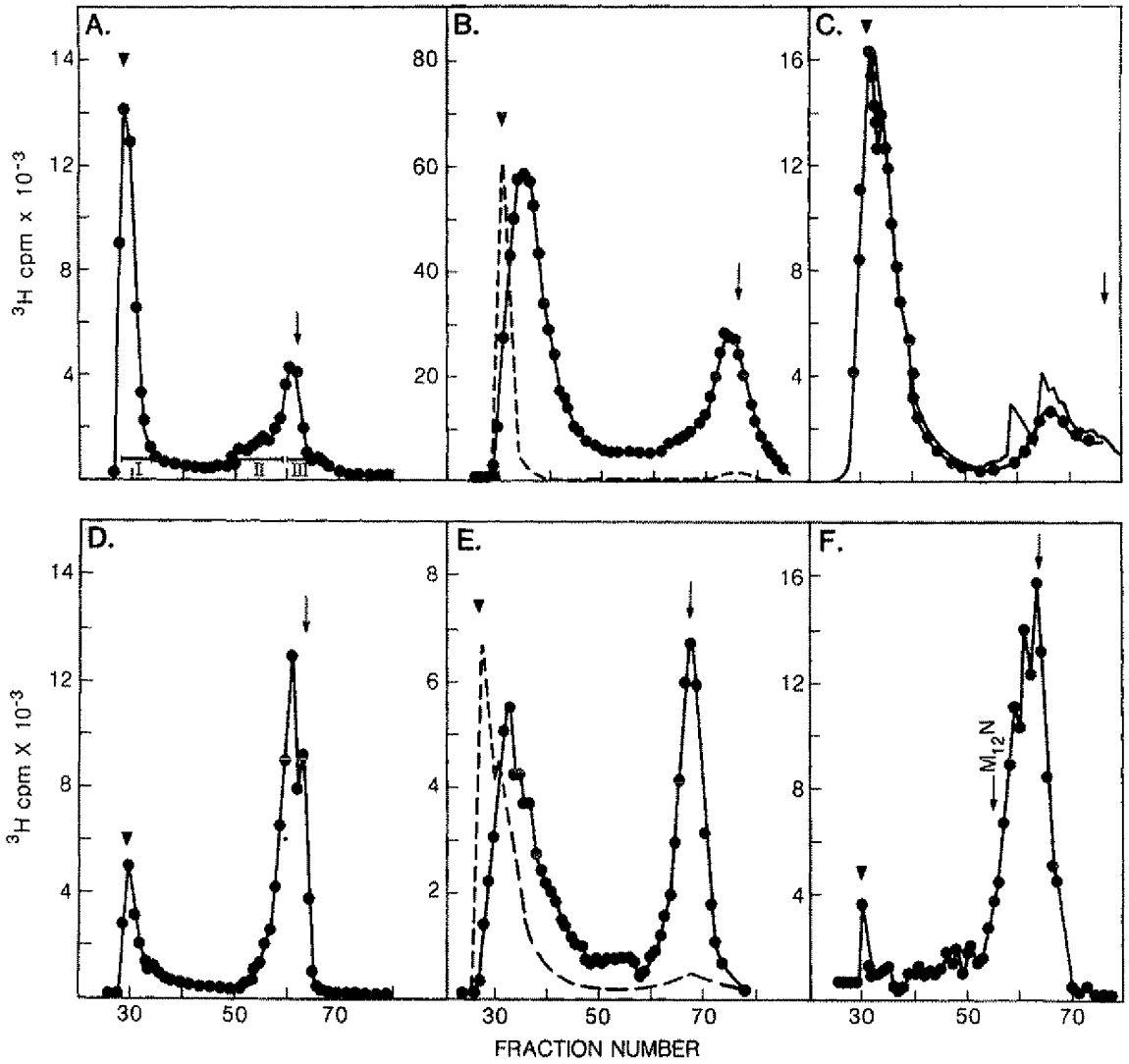

Figure 1. Gel filtration of oligosaccharides isolated from periplasmic glycoprotein of *P. pastoris*. Periplasmic glycoprotein and isolated invertase were prepared and treated as described in Methods. (A) *P. pastoris* periplasmic glycoprotein labelled for 2·5 h and chromatographed on Bio-Gel P-6. Aliquots of 50 µl were counted and fractions were pooled as indicated by bars. (B) *P. pastoris* periplasmic glycoprotein labelled for 24 h (solid line) and *S. cerevisiae* periplasmic glycoprotein labelled for 2·5 h (dashed line) chromatographed on Bio-Gel P-10. (C) *P. pastoris* periplasmic glycoprotein labelled for 24 h treated with Pronase (solid line) and treated with Pronase plus mild alkaline hydrolysis (solid line with dots) chromatographed on Bio-Gel P-6. (D) Oligosaccharides of invertase isolated from *P. pastoris* labelled for 2·5 h and chromatographed on Bio-Gel P-6. (E) Oligosaccharides of invertase isolated from *P. pastoris* labelled for 24 h (solid line) and oligosaccharides of invertase isolated from *S. cerevisiase*, labelled for 2·5 h (dashed line), chromatographed on Bio-Gel P-10. (F) Oligosaccharides of invertase isolated from *P. pastoris* growth media, labelled *in vitro* with NaB[^{3}H]$_4$ and chromatographed on Bio-Gel P-6. Void volume is indicated by ▼ and $Man_5GlcNAc$ is indicated by an arrow.

Structural characteristics of N-asparagine-linked oligosaccharides

Alpha-mannosidase digestions were performed on mannose-labelled oligosaccharides which had previously been purified by gel filtration (Figure 1A, pools I–III). As seen in Figure 2A, α-mannosidase treatment released 15% of the label from the large oligosaccharides of pool I ($Man_{>30}GlcNAc$); however, this release of mannose was insufficient to change the elution behavior of these large oligosaccharides. Pool II ($Man_{10-14}GlcNAc$)

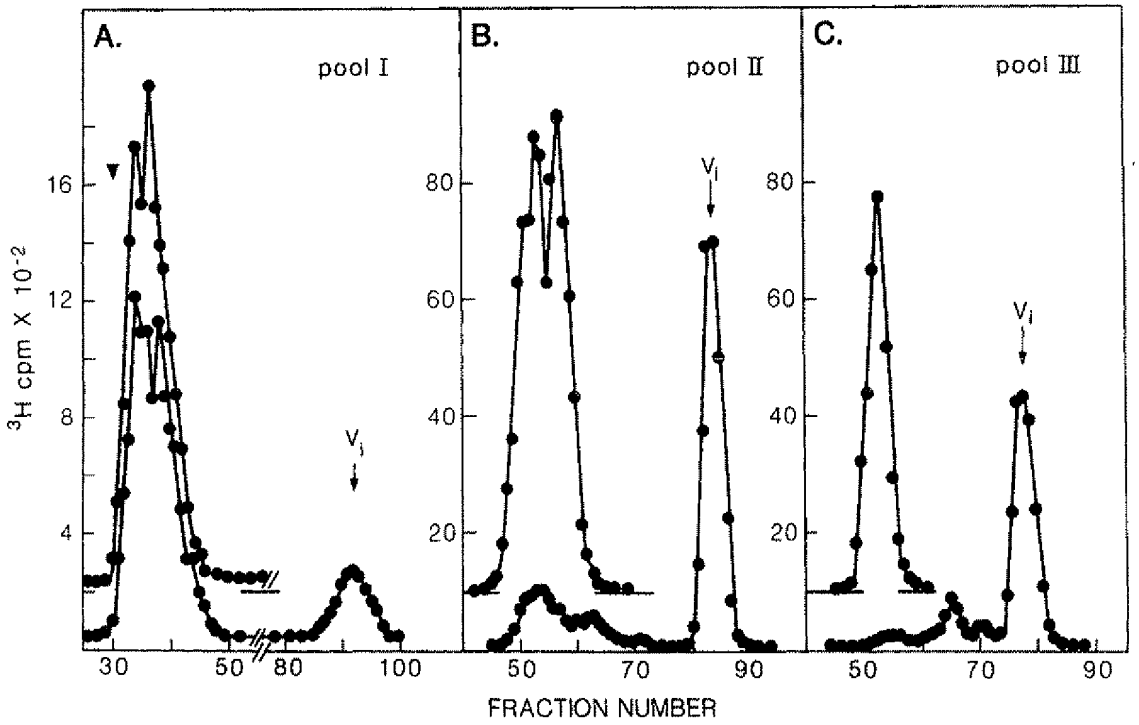

Figure 2. Mannosidase treatment of *P. pastoris* oligosaccharides. Mannose-labelled oligosaccharides were separated into pools (as indicated in Figure 1A), treated with α-mannosidase and fractionated as described in Methods. The upper graphs represent chromatography of the untreated pools and the lower graphs represent chromatography of the α-mannosidase-treated samples. (A) $Man_{<10}GlcNAc$ oligosaccharides fractionated on Bio Gel P-10. (B) $Man_{10-14}GlcNAc$ oligosaccharides fractionated on Bio-Gel P-6. (C) $Man_{8,9}GlcNAc$ oligosaccharides fractionated on Bio-Gel P-4. Where appropriate, the void volume is indicated by ▼.

oligosaccharides were more sensitive to α-mannosidase treatment, and although 21% of the label remained at the original elution position 68% of the label was found as free mannose and 11% was present as oligosaccharides of reduced size (Figure 2B). Oligosaccharides of pool III ($Man_{8,9}GlcNAc$) were completely sensitive to α-mannosidase and were reduced in size to free mannose and very small molecules eluting at or near the inclusion volume of the column (Figure 2C). Similar results were obtained for the oligosaccharides of invertase.

Size-fractionated oligosaccharides (pools I–III) of the periplasmic glycoprotein were used for chromatography on QAE-Sephadex. The oligosaccharides of each pool were separately applied to the anion exchange matrix and eluted stepwise with increasing concentrations of NaCl, thereby giving an indication of the strength of the net negative charge (Trimble *et al.*, 1983; Varki and Kornfeld, 1980). The $Man_{8,9}GlcNAc$ oligosaccharides of pool III were essentially free of net negative charge, and 95% of the mannose-labelled oligosaccharides passed through the QAE-Sephadex without binding (Table 1). The portion which did bind to the matrix represented very little radioactivity and is of dubious significance. When the $Man_{10-14}GlcNAc$ oligosaccharides of pool II were chromatographed on QAE-Sephadex, 32% were bound by the matrix and approximately one-third of these bound oligosaccharides required more than 50 mM-NaCl for elution (Table 1). This may may indicate that some of the pool II oligosaccharides contain more than one negative charge (Trimble *et al.*, 1983; Varki and Kornfeld, 1980). Approximately one-half of the large, mannose-labelled, $Man_{>30}GlcNAc$ oligosaccharides of pool I contained net negative charge and bound to the QAE-Sephadex (Table 1). Although a small amount of the bound oligosaccharide required more than 50 mM-NaCl for elution, the bulk of the bound oligosaccharide was eluted by 10 mM- and 50 mM-NaCl, probably indicating that most of these oligosaccharides contain a single negative charge. The binding of pool I and II oligosaccharides to the QAE-Sephadex is most likely due to the presence of mannose-bound phosphate.

Table 1. QAE-Sephadex chromatography of mannose-labelled oligosaccharides

Elution (mM-NaCl)	$Man_{8,9}GlcNAc$		$Man_{10-14}GlcNAc$		$Man_{>30}GlcNAc$	
	(cpm)	(%)	(cpm)	(%)	(cpm)	(%)
0	8720	95	58 922	68	16 704	52
10	0	0	8089	9	6432	20
50	183	2	8533	10	7736	24
>200	275	3	10 924	13	1293	4

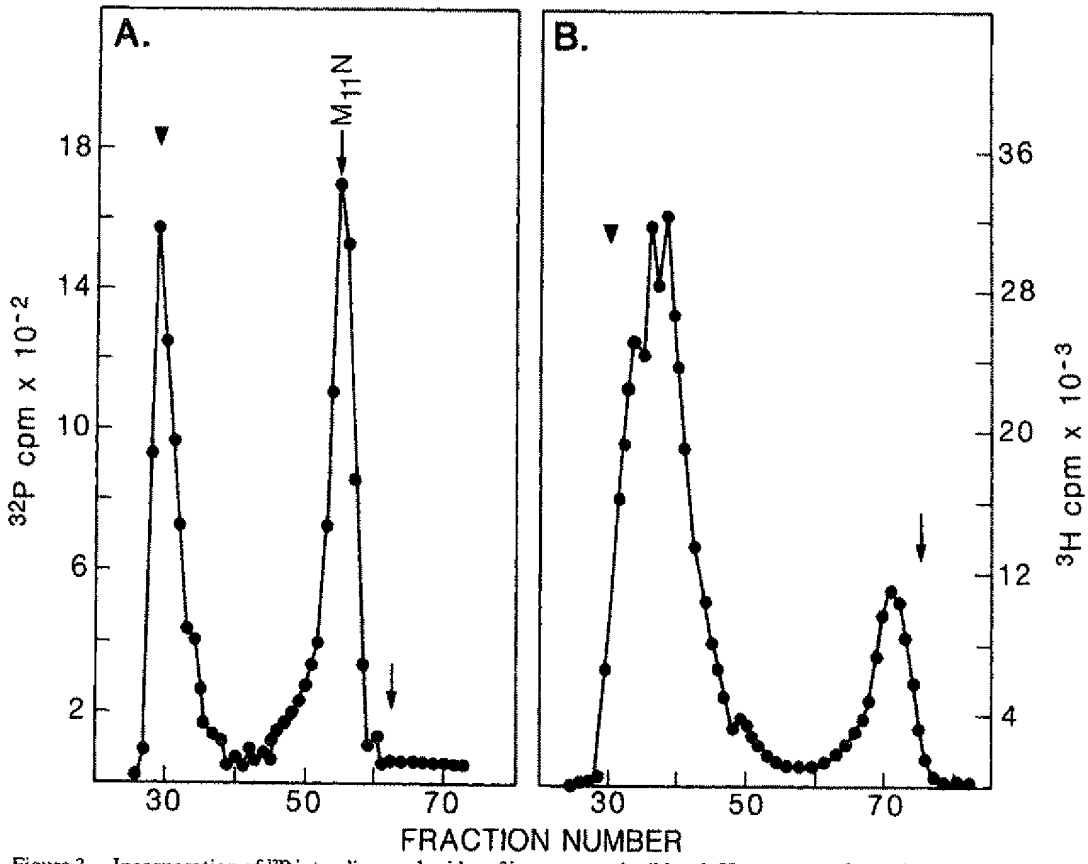

Figure 3. Incorporation of ^{32}P into oligosaccharides of invertase and mild endoH treatment of non-denatured invertase. (A) Oligosaccharides of invertase, isolated from *P. pastoris* growth medium, were labelled *in vivo* with ^{32}P and separated on Bio-Gel P-6 as described in Methods. (B) Mannose-labelled oligosaccharides released from non-denatured invertase. Invertase treated for 5 h with endoH and separated on Bio-Gel P-10. Void volume is indicated by ▼ and $Man_8GlcNAc$ is indicated by an arrow.

To demonstrate the presence of bound phosphate, the oligosaccharides of *P. pastoris* were labelled *in vivo* with ^{32}P. For invertase, the ^{32}P was incorporated in approximately equal amounts in the $Man_{>30}GlcNAc$ oligosaccharides (43%) and in oligosaccharides in the size range $Man_{10-14}GlcNAc$ (57%), but no label was present in the region corresponding to $Man_8GlcNAc$ or $Man_9GlcNAc$ (Figure 3A). This supports results obtained from QAE-Sephadex chromatography which had indicated negative charge on approximately 48% of the large $Man_{>30}GlcNAc$ oligosaccharides and on

approximately 31% of the oligosaccharides in the size range $Man_{10-14}GlcNAc$, but negligible charge on the $Man_{8,9}GlcNAc$ oligosaccharides. Alakaline phosphatase treatment of the ^{32}P-labelled oligosaccharides did not release free ^{32}P, did not alter the binding of the oligosaccharides to QAE-Sephadex, and did not alter the susceptibility of the oligosaccharides to α-mannosidase hydrolysis (data not shown). The phosphate bound to these oligosaccharides is not monoesterified.

Mannose-labelled invertase of *P. pastoris* was immunoprecipitated from media and, without detergent or heat denaturation, was treated by mild endoH digestion. As seen in Figure 3B, small as well as large oligosaccharides were released by the mild treatment. In a series of experiments, endoH routinely released 80–90% of the label associated with the large oligosaccharide fraction and from 20 to 50% of the label associated with the $Man_{8-14}GlcNAc$ oligosaccharide fraction.

DISCUSSION

These studies have demonstrated that *Pichia pastoris* performs *N*-asparagine-linked glycosylation and that the oligosaccharides synthesized are distinctly different in size distribution, and possibly in structure, from those synthesized by *S. cerevisiae*. The glycosylation of heterologous invertase generally reflected the pattern of glycosylation observed for the total soluble glycoprotein secreted by *P. pastoris*.

Glycoproteins secreted by *P. pastoris* contain significant amounts of short-chain oligosaccharides. While *in vivo* mannose labelling experiments indicated that the periplasmic glycoprotein fraction contained more than 35% short-chain oligosaccharides and that heterologous invertase contained from 40 to 70% short-chain oligosaccharides, apparently these estimates are low. The results obtained by *in vitro* sodium borohydride labelling of the oligosaccharides of invertase demonstrated that the short-chain oligosaccharides actually comprised more than 85% of the total oligosaccharide bound to this protein. In contrast, invertase synthesized in *S. cerevisiae* contains only 20% of its mannose in oligosaccharides of size $Man_{8-14}GlcNAc_2$ (Trimble and Atkinson, 1986).

The small oligosaccharides of *S. cerevisiae* and *P. pastoris* do share certain structural features. In *P. pastoris*, as in *S. cerevisiae*, the $Man_8GlcNAc_2$ structure appears to be the ultimate trimming product and is probably the precursor for subsequent elongation reactions (Byrd *et al.*, 1982). Man_8GlcNA_2 and also $Man_9GlcNAc_2$ contain α-linked mannose and are completely sensitive to α-mannosidase. Neither of these structures is labelled by phosphate *in vivo* and neither structure contains net negative charge. The $Man_{10-14}GlcNAc_2$ oligosaccharides, which account for more than half of the total small oligosaccharide fraction, are formed by the addition of two to six mannose to the $Man_8GlcNAc_2$ precursor (Trimble and Atkinson, 1986). The added mannose appears to be α-linked to the core, and the majority of the $Man_{10-14}GlcNAc$ oligosaccharide structures are susceptible to hydrolysis by α-mannosidase. Structures which are resistant to hydrolysis by α-mannosidase account for 21% of the total $Man_{10-14}GlcNAc$ oligosaccharide and presumably are the structures which can be labelled *in vivo* with ^{32}P and which also bind to QAE-Sephadex. The exact size of the α-mannosidase-resistant structures is not known, since the presence of bound phosphate can alter the elution behaviour of oligosaccharides and cause them to appear larger than they are (Trimble *et al.*, 1983). The presence of phosphate on the relatively short-chain $Man_{10-14}GlcNAc$ oligosaccharides of *P. pastoris* is both noteworthy and unusual. On invertase secreted by *S. cerevisiae*, $Man_{10-14}GlcNAc_2$ oligosaccharides do not contain phosphate (Trimble and Atkinson, 1986), and in fact, phosphorylation of oligosaccharides in this size range is characteristic of vacuolar proteins rather than secreted proteins (Stevens *et al.*, 1982). Whether phosphorylation of the small oligosaccharide structures in *P. pastoris* influences processing of these oligosaccharides is not known.

The large oligosaccharides of *P. pastoris* glycoprotein contain more than 30 mannose residues per chain, and although the maximum chain length of these oligosaccharides was not determined, it is evident from comparisons of gel filtration patterns that the largest oligosaccharides of *P. pastoris* are significantly shorter than the extended structures observed for *S. cerevisiae*. The large oligosaccharides synthesized by *P. pastoris* apparently share some structural features with the large oligosaccharides of *S. cerevisiae*. The majority of the oligosaccharides are resistant to hydrolysis by α-mannosidase (Jones and Ballou, 1969), and some of the structures contain diesterified phosphate and will bind with varying degrees of strength to QAE-Sephadex (Trimble *et al.*, 1983). At this time, it is not known whether the large oligosaccharides of *P. pastoris* contain diesterified phosphate in the outer chain, as in *S. cerevisiae* (Ballou, 1982; Trimble and Atkinson, 1986), or whether the

phosphate is present in the inner core region, as observed for the $Man_{10-14}GlcNAc$ oligosaccharides of *P. pastoris*.

The results obtained for invertase allow comparison of the glycosylation of a single, well-characterized protein in two different yeasts. Invertase is synthesized as a 58 000 molecular weight polypeptide in both *S. cerevisiae* and *P. pastoris* (Schekman and Novick, 1982; Tschopp *et al.*, 1987b). In both yeast, an average of nine or ten of 14 available sites are utilized for *N*-asparagine-linked glycosylation (Trimble and Maley, 1977; Tschopp *et al.*, 1987b; Ziegler *et al.*, 1988). On invertase from *S. cerevisiae*, the oligosaccharide chains have variable amounts of carbohydrate, and the extended outer-chain structures may contain more than 50 mannose residues (Tarentino *et al.*, 1974). In contrast, the oligosaccharides of invertase synthesized in *P. pastoris* are more homogeneous in size and average ten mannose resides per chain. While small oligosaccharides on invertase synthesized by *S. cerevisiae* are thought to represent partially processed, intermediate structures and/or oligosaccharides which are buried within the folded molecule (Trimble *et al.*, 1983; Trimble and Atkinson, 1986), in *P. pastoris* the $Man_{8-14}GlcNAc_2$ structures appear both as surface and buried oligosaccharides and apparently represent mature oligosaccharide structures. It is clear that the bulk of the surface-located oligosaccharides of heterologous invertase produced in *P. pastoris* have undergone only minimal processing in their transit of the secretory pathway.

The small and uniformly sized oligosaccharides synthesized by *P. pastoris* are comparable in size, and possibly in structure, to high-mannose oligosaccharides synthesized by animal cells (Kornfeld and Kornfeld, 1985). In contrast, the hypermannosylated oligosaccharides synthesized by *S. cerevisiae* are much larger than the high-mannose oligosaccharides of animal cells. Since these hypermannosylated structures are potentially antigenic (Ballou, 1982), heterologous glycoproteins possessing them would clearly be unsuitable for use in human therapeutic applications. By virtue of its different and distinctive style of glycosylation, *Pichia pastoris* may prove to be a superior host for the production of heterologous glycoproteins.

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